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COMMUNICATIONS

Synthesis, Hybridization Properties, Nuclease Stability, and Cellular Uptake of the Oligonucleotide–Amino-β-cyclodextrins and Adamantane Conjugates

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Synthesis of the oligonucleotides conjugated with amino derivatives of β-cyclodextrin and adamantane, at the 3′-end of host oligonucleotide, has been described. The oligonucleotide conjugates were examined for their nuclease stability, hybridization properties, and cellular uptake. The oligonucleotide conjugates had increased nuclease resistance compared to their parent oligonucleotides. Conjugation of adamantane to the oligonucleotides did not adversely affect the ability of the oligonucleotides to hybridize with their complementary RNA. Conjugation with amino derivatives of β-cyclodextrin, however, significantly destabilized the duplex formation. In the cellular uptake studies, we found that amino derivatives of β-cyclodextrin attached at 3′-end of the oligonucleotides did not help to increase the uptake by cells. Cellular uptake of oligonucleotide–adamantane conjugates in association with 2-(hydroxypropyl)-β-cyclodextrin (HPCD) as a "carrier" was significantly higher than that of control oligonucleotides.

Oligonucleotides are widely used as research tools for inhibiting specific gene expression and are under investigation for possible use as therapeutic agents (*1–4*). Cellular uptake and internalization of such oligonucleotides are important factors in determining their effectiveness as potential therapeutics. Oligonucleotide uptake is a sequence-independent saturable process and is dependent on temperature and energy (*5*, *6*). To improve cellular uptake, several groups of oligonucleotide conjugates have been synthesized and studied (*3*, *7*, *8*). Although some of these conjugates showed increases in cellular uptake and stability against nucleases, cytotoxicity or/and non-sequence-specific activities increased.

In our earlier studies (*9*, *10*) we have explored the possibility of using β-cyclodextrin and its analogues as "carriers" to increase the cellular uptake of oligonucleotides. Cyclodextrins are cyclic oligosaccharides known for their ability to form inclusion complexes with many lipophilic drugs, changing the physicopharmaceutical properties of the drugs such as aqueous solubility, thereby changing their bioavailability and improving stability and effectiveness (*11*, *12*). We demonstrated that cellular uptake of phosphorothioate oligonucleotide was 2–3-fold increased in the presence of cyclodextrin analogues in H9, CEM, and Molt-3 cell lines. The uptake of the oligonucleotide mediated by cyclodextrins was a cyclodextrin concentration and time dependent process, and the intracellular presence of the oligonucleotide was confirmed by confocal microscopy. In this study, we have further explored the possibility of using β-cyclodextrin and its analogues as "carriers" to increase the cellular uptake of oligonucleotides. Because the adamantane molecule can enter into the β-cyclodextrin cavity and form a stable inclusion complex (*13*, *14*), and because cyclodextrin derivatives can act as a selective hosts with several binding sites for nucleotides (*15*, *16*), we decided to link a molecule of adamantane or a molecule of the amino derivatives of β-cyclodextrin at the 3′-end of a synthetic oligonucleotide.

A molecule of adamantane was attached to an oligonucleotide via an amino linker. 3-Aminopropyl solketal **1** was synthesized as described (*17*) and further reacted with 1-adamantanecarbonyl chloride (Scheme 1) to give *N*-adamantoyl-3-(aminopropyl)solketal (**2**) with a yield of 97%. Adamantoyl derivative **2** was treated with a

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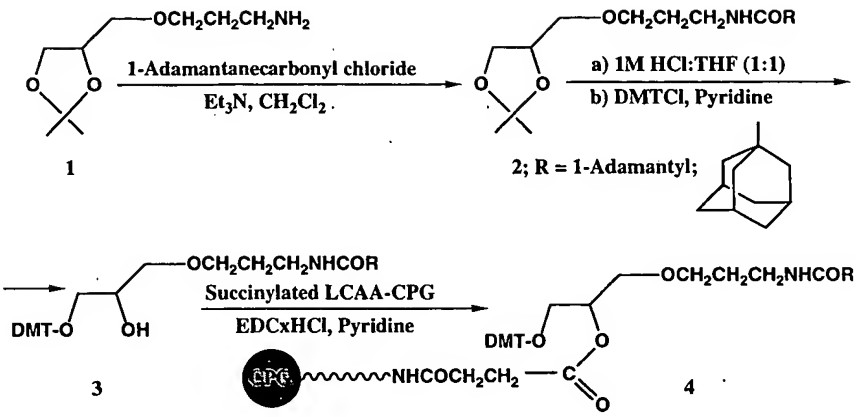
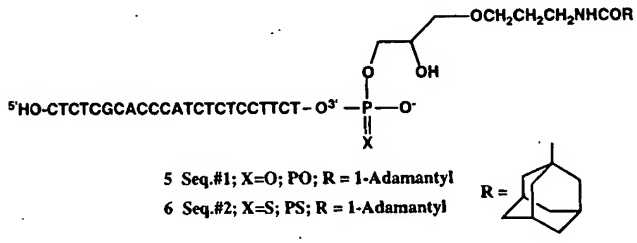
Scheme 1



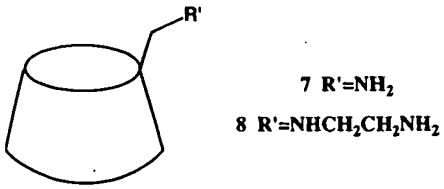
mixture of 1 M hydrochloric acid and tetrahydrofuran (1:1) to remove the isopropylidene group and *in situ* reacted with 4,4′-dimethoxytrityl chloride in anhydrous pyridine to give 1-*O*-(4,4′-dimethoxytrityl)-3-*O*-(*N*-adamantoyl-3-aminopropyl)glycerol (**3**) with a yield of 61% (Scheme 1). The DMT derivative **3** was further attached (*18*) onto long chain (alkylamido)propanoic acid controlled pore glass (LCAA-CPG) beads to give **4** and was as such used for oligonucleotide synthesis. The loading efficiency was 22.1 μmol/g of CPG.

Synthesis of the oligonucleotides **5** and **6**, containing either phosphodiester (PO) or phosphorothioate (PS) internucleoside linkages, respectively, was carried out on 10 μmol scales using **4** and β-cyanoethyl 3′-phosphoramidites on an automated DNA synthesizer (Milligen/Bioresearch 8700 series). The oxidation reagents used



for the synthesis were a standard solution of iodine for PO linkage formation and a 1% solution of 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in acetonitrile for PS linkage formation. Oligonucleotides **5** and **6** were released from the support by treatment with ammonia for 6 h at 55 °C and purified by reversed-phase HPLC at pre- and post-DMT removal stages (Figure 1).

Amino derivatives of β-cyclodextrin (**7** and **8**) were prepared as previously described (*19*, *20*) and attached at the 3′-end of the oligonucleotides via carbamate (−OCONH−) linkage (*21*−*23*) (Scheme 2). Synthesis of oligonucleotides **9** and **10** was carried out on 1 μmol scale using β-cyanoethyl 5′-phosphoramidites on an automated DNA synthesizer with the last DMT removed. The 3′-OH group was further activated with bis(*p*-nitrophenyl)-carbonate in anhydrous 1,4-dioxane with triethylamine as the catalyst (3 drops) for 2 h to give the active



7 R'=NH_2

8 R'=$NHCH_2CH_2NH_2$

carbonates **11** and **12** (Scheme 2). The active oligonucleotide carbonates were then successively washed with anhydrous 1,4-dioxane and acetonitrile, dried by purging with argon, and reacted with **7** or **8** in anhydrous pyridine for 6 h to give **13**−**16**. After successive washings with pyridine and acetonitrile, oligonucleotides were released

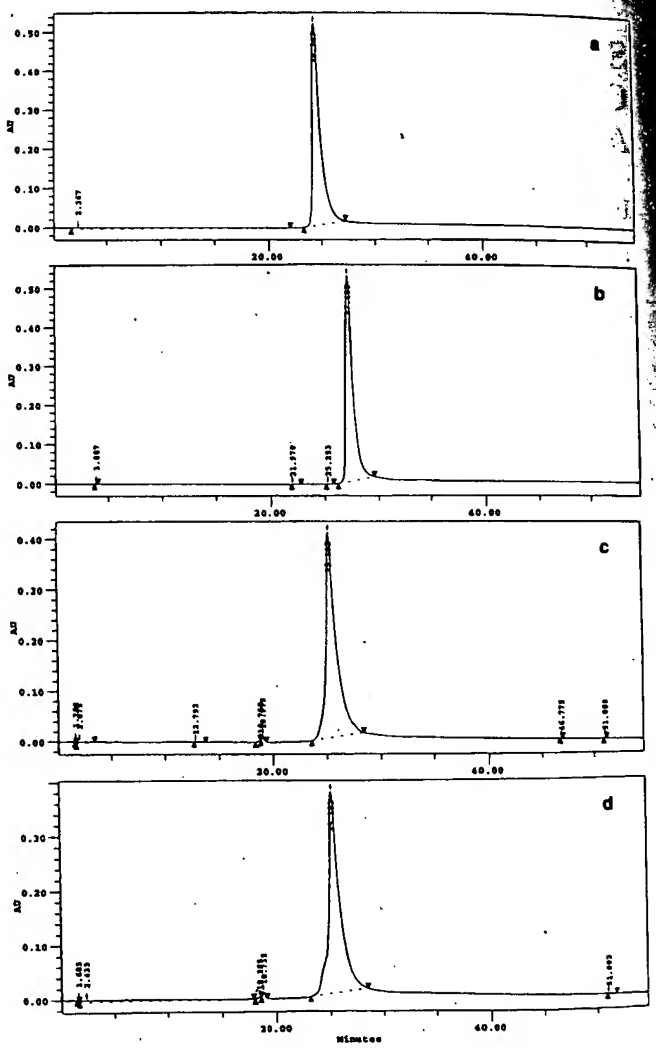


Figure 1. Reversed-phase HPLC profile of oligonucleotides. (a) Standard PO oligonucleotide with the same chain length and base composition as **5**, **17**, and **18** and with no modification at 3′-end; (b) oligonucleotide **5**; (c) **17**; (d) **18**. HPLC was carried out using Waters 600E system controller, 996 Photodiode array detector, NEC PowerMate 486/33i, and Millenium 2010 chromatography manager. For reversed-phase HPLC, the column used was Radial-Pak Cartridge (Waters), buffers (A) 0.1 M ammonium acetate and (B) buffer A containing 80% acetonitrile; gradient 0% buffer B for 2 min, 0−20% buffer B for 30 min, flow 1.5 mL/min, detector 254 nM.

Scheme 2

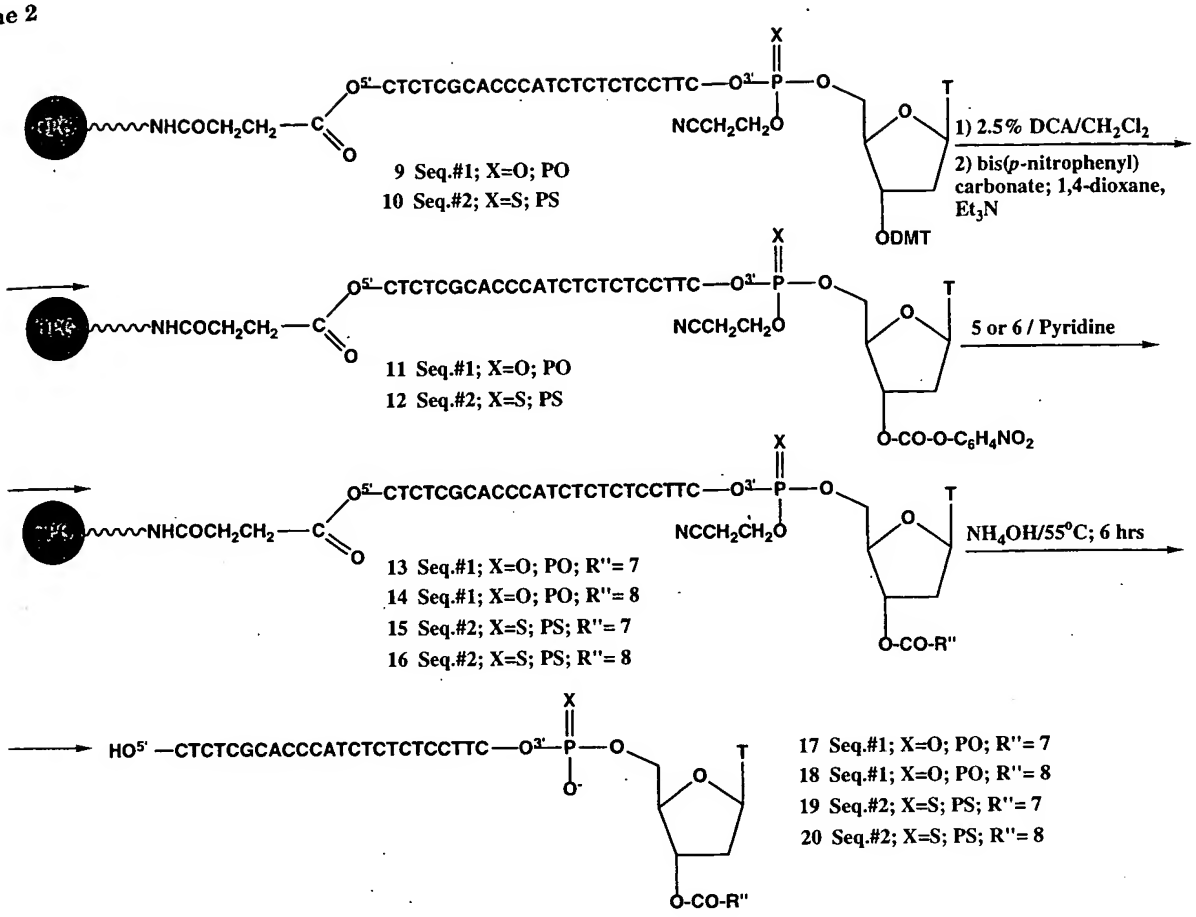


Table 1. Hybridization Data of Oligonucleotides[a]

Entry	Oligonucleotide	Tm[b], (°C)	Tm[c], (°C)	ΔTm, (°C)
1	5; PO; Oligonucleotide—O3'—P(O)(O⁻)—O—CH(CH2OH)—CH2—OCH2CH2CH2NHCO—Adamantyl	70.6	70.0	-
2	6; PS	62.5	62.3	-
3	17; PO; Oligonucleotide—O3'—CO—NH—Adamantyl	42.9	70.0	-27.1
4	19; PS	36.0	62.3	-26.3
5	18; PO; Oligonucleotide—O3'—CO—NHCH2CH2NH—Adamantyl	42.3	70.0	-27.7
6	20; PS	35.8	62.3	-26.5

[a]Oligonucleotides were hybridized with complementary RNA; [b]Absorbance vs temperature profiles were measured at 0.2 A_{260} Units of each strand in 1 mL of buffer (100 mM Na^+, 10 mM phosphate, pH 7.0); [c]Standard PO and PS oligonucleotides with the same chain length and sequence as the oligonucleotides 5, 6, 17-20, and with no modification at the 3'-end.

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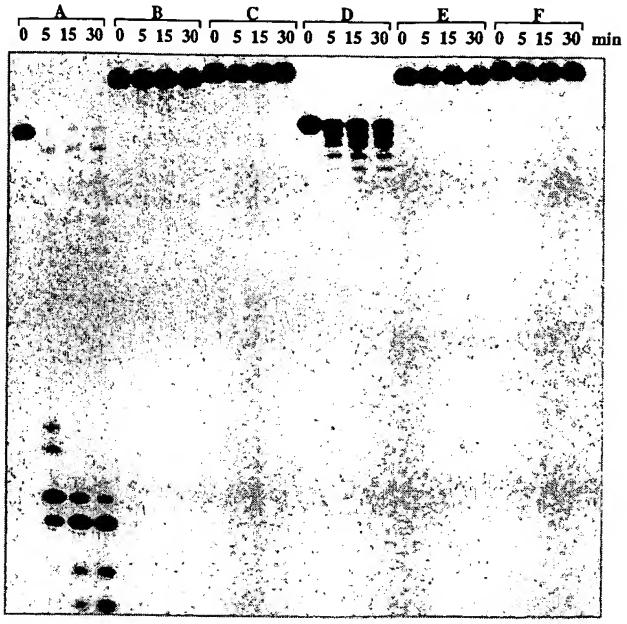

Figure 2. PAGE of enzymatically digested oligonucleotides with the amino β-cyclodextrins covalently linked at the 3′-end. The standard oligonucleotides (25-mers), with the same sequence as oligonucleotides **17**–**20**, containing all PO linkages (A) and all PS linkages, (D); oligonucleotides **17** (B), **18** (C), **19** (E), and **20** (F) were labeled (*25*) with [γ-^{32}P]ATP. T4 DNA polymerase resistance was studied by incubating 30 pmol of each oligonucleotide with 5 units (≥5 units/mg) of the enzyme at 37 °C in 20 μL of buffer (50 mM Tris, pH 8.0, 5 mM Mg^{2+}, 5 mM DTT, 0.05% bovine serum albumin). At the indicated time, an aliquot (5 μL) was removed, and to each aliquot stop solution (6 μL; 95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added. The samples were then analyzed by electrophoresis on 20% polyacrylamide gel containing 8 M urea.

from the support, deprotected by treatment with ammonia for 6 h at 55 °C, and purified by polyacrylamide gel electrophoresis to give oligonucleotides **17**–**20** (Figure 1).

Hybridization studies indicated that attachment of a molecule of adamantane via an amino linker at the 3′-end of oligonucleotides, **5** and **6**, does not affect the stability of the duplexes formed between oligonucleotides and their complementary RNA (Table 1, entries 1 and 2). The same behavior in the hybridization was previously observed with an oligonucleotide with a molecule of adamantane attached at 3′-end via a phosphorothioate linkage (*24*). The attachment of amino derivatives of β-cyclodextrin, **7** or **8**, via a carbamate linkage at the 3′-end of oligonucleotides **17**–**20** caused a significant reduction in binding (Table 1, entries 3–6). This poor binding could be related to complexation properties (*15*, *16*) of nucleotidic units of an oligonucleotide and an amino derivative of β-cyclodextrin that is attached to the host oligonucleotide. In addition, β-cyclodextrin is a sterically crowded molecule. A combination of these factors resulted in only partial duplex formation of oligonucleotides **17**–**20** with the complementary RNA (Table 1, entries 3–6).

We determined the nuclease resistance of oligonucleotides **17**–**20** to a 3′-exonuclease, such as T4 DNA polymerase (Figure 2). The kinetic study showed complete degradation of PO oligonucleotide (25-mer, with the same sequence as **18** and **19**) in less than 5 min, while the great majority of PO oligonucleotides **18** and **19**, which had amino derivatives of β-cyclodextrin **7** and **8** attached at their 3′-ends, remained intact even after 30 min. The PS oligonucleotide exhibited better stability

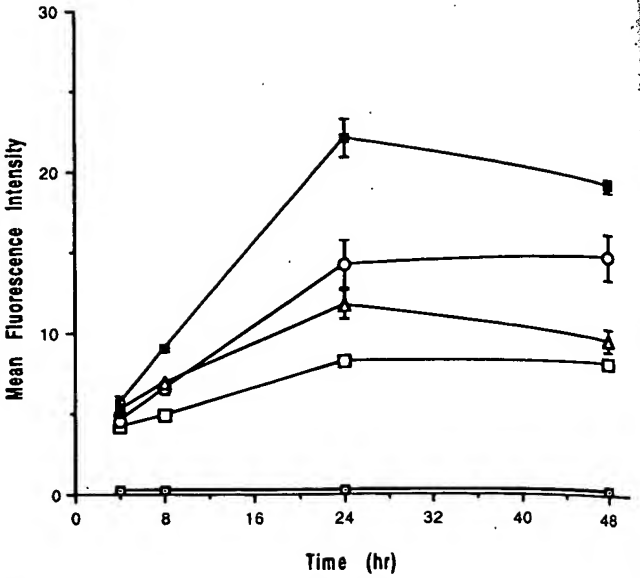

Figure 3. Cellular uptake profiles of oligonucleotides mediated by 2-(hydroxypropyl)-β-cyclodextrin (HPCD). Oligonucleotide **6** and HPCD (−■−); oligonucleotide **6** (−△−); PS oligonucleotide (25-mer, the same sequence as **6**) (−□−); PS oligonucleotide & HPCD (−○−); and media without oligonucleotide (−⊡−). Each oligonucleotide was mixed with 1.25% solution of HPCD (sterilized prior to use by passing through 0.2 μm filter) in plain RPMI media to give a concentration of 10 μg of oligonucleotide *per* mL of media, sonicated at 4 °C for 2 h, and followed by complexation at 4 °C overnight. H9 cells were cultured in RPMI media supplemented with 10% heat inactivated (56 °C for 30 min) fetal bovine serum (FBS), 2 mM glutamine, 10 μg/mL of streptomycin, 100 units/mL of penicillin, and 50 μM mercaptoethanol at 37 °C in a humidified air incubator (5% CO_2–95% O_2). Each fluorescein-labeled oligonucleotide alone or as HPCD complex was separately mixed with H9 cells (10^6 cells/mL) in the 1:1 (v/v) ratio and incubated at 37 °C in incubator. At various time points, ranging from 2 to 48 h, the aliquots were removed from each mixture, washed, and resuspended in Hank's balanced salt solution (HBSS) supplemented with 0.1% bovine serum albumin (BSA) and 0.1% sodium azide. Propidium iodide staining was used to distinguish viable cells from dead cells. Flow cytometric data on 5,000 viable cells was acquired in listmode on Epics XL flow cytometer and data were analyzed by Epics XL version 1.5 software after gating on living cells by forward scatter vs side scater and propidium iodide staining.

under the same conditions than the PO oligonucleotide, which is expected, and the PS oligonucleotides **19** and **20** showed no sign of degradation even after 30 min (Figure 2).

To determine the usefulness of cyclodextrins as "carriers" for oligonucleotides which were labeled (*26*) with fluorescein at the 5′-end, we screened a series of cyclodextrins and their analogues (*9*, *10*). In the studies involving oligonucleotide–adamantane conjugate **6**, 2-(hydroxypropyl)-β-cyclodextrin (HPCD) was the most efficient carrier (Figure 3). In these experiments the same oligonucleotide **6** as a non-HPCD complex, PS oligonucleotide (25-mer, the same sequence as **6** but without adamantane) as an HPCD complex, or alone were used as the controls (Figure 3). We observed the highest level of oligonucleotide uptake with oligonucleotide **6** as HPCD complex. The lowest cellular uptake was exhibited by the control PS oligonucleotide alone and was improved by HPCD complexation (Figure 3). More significant changes in uptake between oligonucleotides were observed after 8 h of incubation reaching the maximum at 24 h, with no significant changes in the following 24 h.

We also studied PS oligonucleotides **18** and **20** fluorescein-labeled (*26*) at the 5′-end, with the amino derivatives of β-cyclodextrin attached at 3′-end, using the same

conditions as described for the above oligonucleotides. They exhibited no higher cellular uptake than did the standard PS oligonucleotide. Previous studies suggest that cellular uptake of the oligonucleotides mediated by cyclodextrins is dependent on the concentration of the cyclodextrin (*9*, *10*). To observe changes in uptake it is necessary to have $7-(7 \times 10)^3$-fold excess in molar ratio of cyclodextrin to that of oligonucleotide. There is no parallel between uptake of the oligonucleotides conjugated with and the oligonucleotides associated with cyclodextrins; however, the results presented here show that one molecule of cyclodextrin attached to an oligonucleotide does not make a difference in cellular uptake.

We have successfully synthesized oligonucleotide–amino-β-cyclodextrins and oligonucleotide–adamantane conjugates. Although oligonucleotides with amino derivatives of β-cyclodextrin attached at the 3′-end exhibited a significant increase in stability against 3′-exonucleases, duplex formation with complementary RNA was destabilized, and we observed no increase in cellular uptake compared to standard oligonucleotide. Oligonucleotide–adamantane conjugates associated in complex with 2-(hydroxypropyl)-β-cyclodextrin (HPCD) also showed higher stability against 3′-exonucleases than standard PS oligonucleotides and increased cellular uptake and had no negative effects on duplex stability. This latter treatment is therefore attractive for use in studies of gene-expression. Oligonucleotide–adamantane conjugates might also better cross the blood–brain barrier (BBB), as was demonstrated with an AZT–adamantane prodrug complex (*27*). The oligonucleotide–adamantane conjugates are presently being studied for their gene-regulation activity.

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Supporting Information Available: Listings of synthetic procedures and spectral data for all new compounds (10 pages). Ordering information is given on any current masthead page.

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